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(2)

ENVIRONMENTAL FATE AND BIOLOGICAL CONSEQUENCES OF CHEMICALS RELATED TO AIR FORCE ACTIVITIES

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Intact soil cores	Biodegradation

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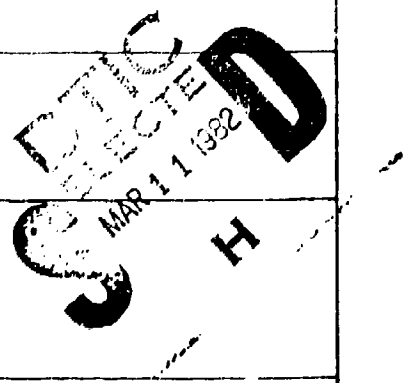

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1. INTRODUCTION

1.1 BACKGROUND

Many man-made chemicals or chemicals related to man's activity are potential hazards to human health and to the welfare of other biological organisms when released into natural terrestrial and aquatic ecosystems. The quantity of material and its degree of toxicity, mobility, persistence, and bioaccumulation are some of the significant factors for consideration in the assessment of potential damage to human health by chemicals. Sources of materials foreign to natural ecosystems include accidental spills, deliberate dumping, hazardous waste disposal, and deliberate application as in military action and application to crops.

This research program is designed to develop methodology to aid in the prediction of the fate and biological effects of released foreign materials on soil surfaces and to assess their fate and migration through the soil and groundwater. The ultimate goal is to develop a valid approach using a laboratory system to assess the fate and effects of test materials before they are widely used in the environment.

1.2 OBJECTIVES

The specific objectives of this research program are to:

- Develop a valid protocol for assessing the environmental effects of chemicals related to Air Force activities in a laboratory terrestrial ecosytem to provide information for preparation of effective environmental impact statements.
- Develop and evaluate a laboratory model terrestrial ecosystem that can be used to determine the effects of these materials on soil biota.
- Develop in situ sampling systems that will permit the efficient recovery of degradation products and metabolites as a test material progresses through an intact soil core in the ecosystem.
- Assess the bioactivity of these products and metabolites for health and ecological effects as the test material moves through the model ecosystem.
- Chemically characterize the bioactive components by instrumental analytical methods.
- Correlate the resulting data using computer methods.
- Finally, validate the test system by field studies.

In the first year of study [1], terrestrial ecosystems were designed and fabricated and preliminary evaluations of these systems were made. Sampling probes and inserts were made and tested, absorbent materials were evaluated, toxicity and mutagenicity tests of recovered organic compounds in leachates were selected and evaluated, column equilibration techniques were studied, and a testing protocol for evaluating test materials was established.

The highlights of this past year of effort include:

- Modification and refinements of terrestrial ecosystems.
- Modification of sampling probes.
- Modification of core-filling techniques.
- Testing of organic compound extraction procedures.
- Improvement of gas chromatographic techniques for organic compound analyses.
- Analysis of headspace vapors from treated ecosystems.
- Analysis of hydrocarbons recovered from leachate from treated soil cores.
- Initiation of biota recovery studies and evaluation of techniques.
- Study of biodegradation of model JP-5 fuel by soil microflora in reaction flasks.
- Further evaluation of toxicity of jet fuels using in vitro mammalian cell techniques.

This report also describes the modification of equipment, experimental procedures, and the data recovered from evaluation of the soil ecosystems using JP-4 jet engine fuels. While reading this report, it should be kept in mind that it is an interim report and much of the data is incomplete, therefore no definitive conclusions can be made at this time.

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[1] Ross, W. D., Hillan, W. J., Wininger, M. T., McMillin, C. R., Gridley, J. A., Kebe, S. C., Aubuchon, J. J., Spillman, J. E., Gohmann, C. M., and Hughes, G. A. Environmental fate and biological consequences of chemicals related to Air Force activities. Dayton, Ohio; Mosanto Research Corporation; 1980 September. 33p. Contract F49620-79-C-0207, Report No. MRC-DA-1000.

2. EXPERIMENTAL

Considerable research has been performed by other researchers using variations on laboratory terrestrial ecosystems (see the bibliography of the First Annual Report, reference 1). Many of the test systems and procedures use standardized soils, biota and meteorological conditions. Because of the complexity and variety of different soil and water ecosystems in nature, we have chosen to investigate the problem using a more empirical approach where the actual site of potential spills is simulated in the laboratory. This is done by acquiring soil cores and meteorological history from the site of concern. These experimental conditions are used to mimic the field conditions as closely as possible.

2.1 MODIFICATION OF ECOSYSTEM DESIGN

Figure 1 is an illustration of the laboratory terrestrial ecosystem as developed in the first year of research. Several minor modifications were made this year as a result of needs identified in initial testing of the apparatus.

2.1.1 Sampling Probes and Sampling Boats

The sampling probes of the latest ecosystem design, which are used both to recover aqueous leachate by channeling leachate outside of the ecosystem and for insertion of the sampling boat inserts, have been modified by placing the probes at a slight downward angle (10° below horizontal) to facilitate immediate flow of leachate to the sampling bottles (not shown Figure 1). Prior to this change, leachate would occasionally fill up the probes because of surface tension before running out, causing misreading of leachate migration rate data.

Modification of the sampling probe and inserts were also made, as shown in Figure 2. This modification (Fig. 2B) addressed a need for the option of permitting aqueous leachate to pass through the adsorbent for recovery of organic compounds without impeding the flow of water to lower soil depths. The initially designed probe (Figure 2A) permits shunting of the leachate laterally to the outside for monitoring flow rates and volumes. Depending on the particular study required, either option is now available.

2.1.2 Reduction of Headspace Volume

In preparation for monitoring headspace vapors above the soil cores, one ecosystem was shortened to reduce headspace. The original 100-cm tube was cut to 75-cm. This modified ecosystem will aid in concentration of the volatile components into a smaller headspace volume. Volatile degradation products or volatile components of the test material can then be recovered and analyzed.

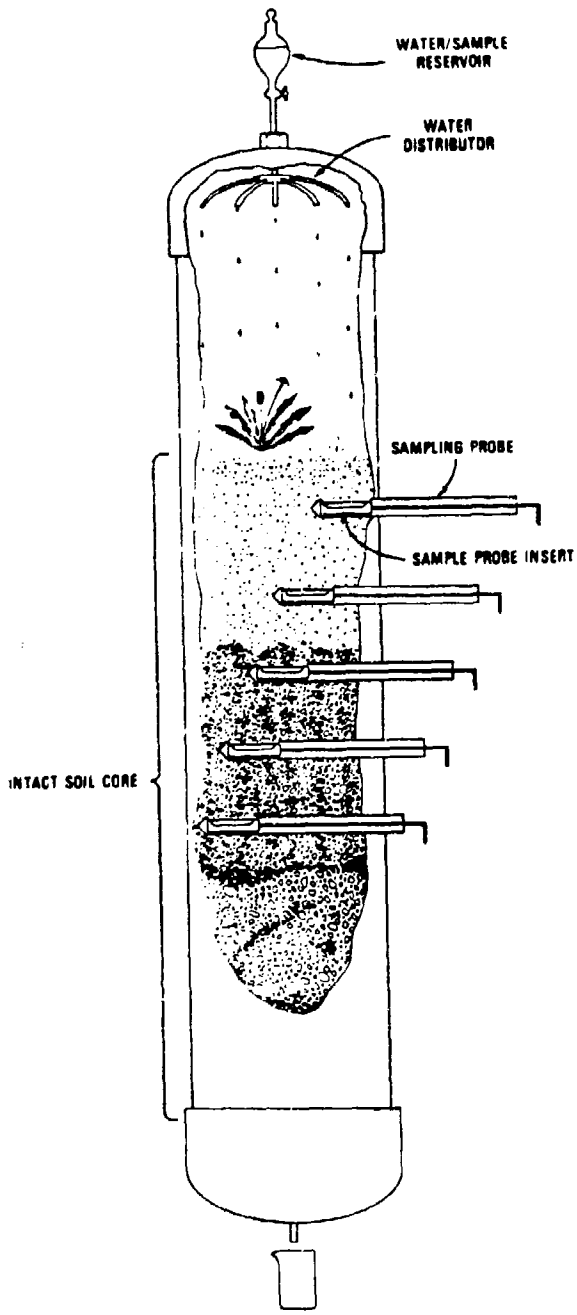
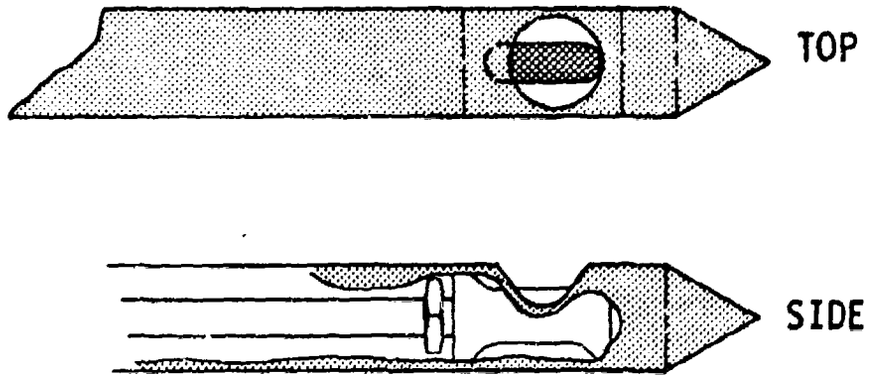
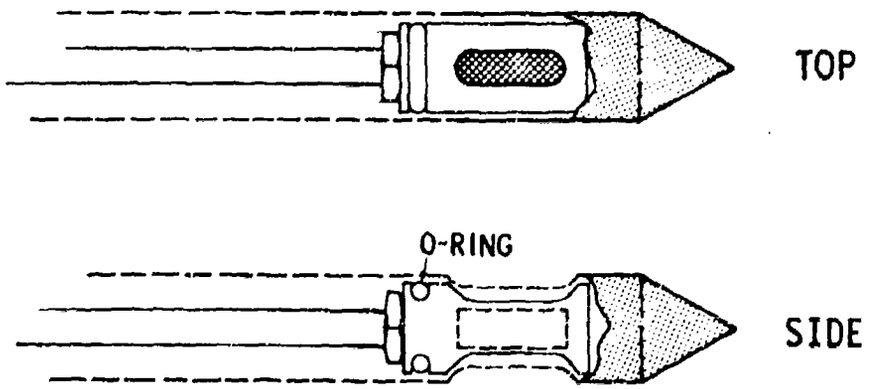


Figure 1. Laboratory terrestrial ecosystem.



A. INITIAL DESIGN



B. MODIFIED DESIGN

Figure 2. Illustration of sampling probe, original and modified designs.

2.1.3 Headspace Vapor Recovery

The headspace vapors and gases can be recovered by a minor modification of the ecosystem. The top inlet port, a 1/4 inch (0.6 cm) male stainless steel Swagelok fitting, was fitted with a Teflon silicone septum. A 1/4-inch (0.6 cm) female fitting is coupled firmly over the septum. A gas syringe is inserted through the septum and headspace gases are removed for subsequent injection into the gas chromatograph. (See Section 2.2.6 for a discussion of headspace gas analysis).

2.1.4 Groundwater Simulation

Variations in groundwater could possibly affect lateral and vertical transport of test material through the soil. Studies were undertaken to mimic groundwater variations in the laboratory test systems by maintaining a reservoir of water adjacent to the ecosystems. A tube running from the bottom of the reservoir to the bottom part of the ecosystem is used to supply subsurface water to the soil core. Groundwater levels are changed by raising and lowering the water reservoir, simulating groundwater fluctuations.

2.2 ANALYTICAL METHODOLOGIES

The analytical methodologies for assessing migration rates, changes in compounds, headspace gas, formation of new metabolites, leachate components, and changes in biota are all end points in assessing the fate and effects of test materials as they progress through the soil cores. The techniques used are described.

2.2.1 Gas Chromatography

Gas chromatography is used to follow the migration of and changes in hydrocarbon fuels as they progress through the ecosystem. This technique is also used for analyzing ecosystem headspace gases. Figure 3 is a chromatogram of shale-derived JP-4 jet fuel. The chromatographic conditions used to analyze this complex hydrocarbon mixture are listed in Table 1.

2.2.2 Carbon Dioxide Analysis

Carbon dioxide in the headspace gases is measured to assess the equilibration of cores, ie., stabilization to laboratory conditions, prior to treatment with a test compound. Carbon dioxide evolution is an indicator of biota activity and equilibration is assumed when a constant evolution is indicated. Carbon dioxide is also measured after treatment of the cores with test materials to note changes in headspace content, which is used as an indicator of stress to the biota or changes in metabolic activity of the soil.

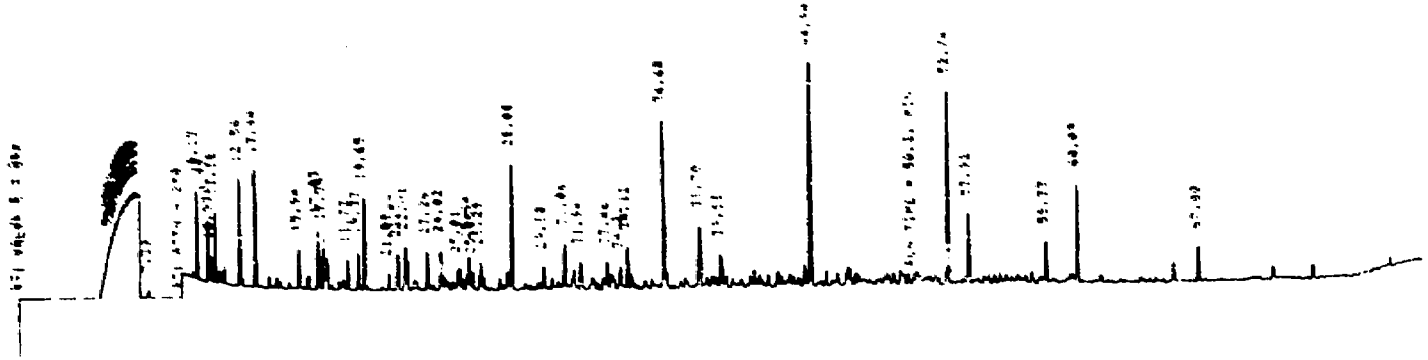


Figure 3. Gas chromatogram of JP-4 jet fuel.

TABLE 1. GAS CHROMATOGRAPHIC INSTRUMENT CONDITIONS USED IN JET FUEL ANALYSES

Instrument:	Hewlett-Packard 5880
Carrier flow:	1.2 mL/min
Column:	50 x 0.2 mm I.D. fused silica
Liquid phase:	dimethyl silicone fluid
Detector:	flame ionization
Oven temperature:	5°C → 155°C at 2°C/min

The analytical procedure used for the analysis of headspace carbon dioxide [2] involves recovery of the CO_2 in 10-mL alkali traps containing 5 mL of 0.8 N potassium hydroxide. These traps were suspended in the headspace of the ecosystems. The traps are removed periodically and titrated with 0.3 N hydrochloric acid.

2.2.3 Standard Test Materials

Two test materials are being used to evaluate the test procedures and to develop the terrestrial ecosystems: jet engine fuels, shale-derived JP-4, and a model JP-5 fuel that theoretically contains equimolar amounts of sixteen known compounds found in JP-5 fuel (see Table 2). Two components were unavailable at the time when the standard was made, cis and trans decalin. Therefore, our model JP-5 contains 14 components. This model test mixture is used by another contractor to study sorption of Air Force fuels by sediment. The Air Force also is interested in determining fate of jet fuel in soil systems. Therefore, model JP-5 will be evaluated in the terrestrial ecosystem in future studies.

The mixture of known hydrocarbon compounds can readily be gas chromatographed (see Figure 4, standard). Hence, the sample can be easily traced as it progresses through the soil ecosystem by gas chromatographic analyses. The standard mixture was also used in a preliminary biodegradation experiment, which is described in section 2.2.4 below.

2.2.4 Biodegradation of Model Jet Fuel

An assessment of degradation of jet fuels by soil microbes was performed in culture flasks in order to determine the role of biota versus chemical and physical degradation in soil ecosytems. Model JP-5 jet fuel was used as a standard hydrocarbon mixture. Changes in known compounds and component quantities as a result of the microbial degradation were assessed by gas chromatographic techniques.

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[2] Titrimetric method for free carbon dioxide. In: Standard Methods for the examination of water and wastewater, 14th edition, 1975. Washington, American Public Health Assoc., 1976, 298-300.

TABLE 2. MODEL JP-5 COMPONENTS

Name	Empirical formula	Molecular weight	Boiling point (°C)	Melting point (°C)	Density (d_4^{20})	Amount added
1,3,5-Trimethylbenzene	C_9H_{12}	120.19	164 - 165	-	0.8637	13.9mL
n-Decane	$C_{10}H_{22}$	142.29	174	-	0.7300	10mL[a]
cis-Decalin	$C_{10}H_{18}$	138	195.7	-	0.8963	15.4mL[b]
trans-Decalin	$C_{10}H_{18}$	138	187	-	0.87	15.9mL[b]
n-Undecane	$C_{11}H_{24}$	156	196	-	0.74017	21.0mL
1,2,4,5-Tetramethyl-benzene	$C_{10}H_{14}$	134	-	80 - 82	0.84[c]	16.0g
1,2,3,4-Tetramethyl-benzene	$C_{10}H_{14}$	134	204 - 205	-	0.901	14.9mL
1,2,3,4 Tetrahydro-naphthalene	$C_{10}H_{12}$	132	207		0.9702	13.6mL
Naphthalene	$C_{10}H_8$	128		79 - 81	1.162	12.8g
n-Dodecane	$C_{12}H_{26}$	170	215		0.7487	22.7mL
2-Methyl naphthlene	$C_{11}H_{10}$	142		32 - 34	1.029	13.8mL
n-Tridecane	$C_{13}H_{28}$	184	234		0.7559	24.2mL
Biphenyl	$C_{12}H_{10}$	154	254	69 - 71	1.041	14.8mL
2,6 Dimethyl-naphthalene	$C_{12}H_{12}$	156		108 - 110	1.008	15.6g
n-Pentadecane	$C_{14}H_{32}$	212	270		0.7689	27.6mL
2,3 Dimethyl naphthalene	$C_{12}H_{12}$	156		102 - 104	1.008	15.6g

[a] 10mL is not the equimolar amount for this compound; 19.5 mL is.

[b] cis- and trans-decalin were not available when this model compound was prepared.

[c] d_4^{81}.

2.2.4.1 Procedure

Soil inoculum was prepared from 500 grams of soil (see section 2.3.1 for soil description) and 1 liter of chlorine-free, autoclaved tap water. The soil-water mixture was shaken and then permitted to stand until particles settled. The water was filtered through glass wool. Two tenths milliliter of this inoculum was added to 500 mL of basal medium in growth flasks. A standard sample of n-dodecylbenzene sulfonate sodium salt (DBSS) (Pfaltz and Bauer) at a concentration of 40.3 mg/mL was prepared. This is equivalent to 12.5 mg of carbon per 500 milliliters of medium. Standard DBSS (0.5 mL) and 0.2 mL of soil microbial inoculum was added to a growth flask (Flask A) containing 500 mL of basal medium. Model JP-5 (16.25 µL) was then added to 500 mL of basal medium, along with 0.2 mL of inoculum, to another growth flaks (Flask B). Model JP-5 (130 µL) was added to a third flask (Flask C) containing 0.2 mL inoculum and 500 mL basal medium. Four adaptive transfers were made at two-day intervals; 1 mL of the contents of Flasks A, B, and C were transferred to fresh 500 mL basal medium containing the same amounts of carbon sources as in the original flasks. A control (Flask D) was carried through with the positive carbon flasks.

Yeast extract was added to all growth flasks as a supplemental growth factor (1 mL/flask of 15 mg/100 mL). Table 3 summarizes the experimental regimen. On the designated transfer days, all growth flasks were shaken vigorously by hand. Three milliliters of the contents of flasks A_1, A_2, A_3 and D were analyzed colorimetrically for DBSS content. Ten milliliters of the B and C series were extracted with 5 mL pentane in preparation for gas chromatography of model JP-5 (See chromatograms in Figures 4-11).

2.2.4.2 Analytical Methods

At each sampling time, 0.1 mL of each biodegradation culture was cultured on nutrient agar by adding it to the agar surface and streaking in three directions. The culture dishes were incubated at 37°C for 24 to 48 hours and then observed for relative amounts of colony growth and types of colonies.

Sampling, extraction, and analysis were performed sequentially over a 25-day period to analyze for indications of microbial degradation of the fourteen components of our model JP-5 listed in Table 2. The extraction procedure involved shaking 10 mL of medium from each fermentation flask with 5 mL of pentane. A 2 µL portion of this extract was analyzed by gas chromatography using the instrument conditions listed in Table 2. The sequence of chromatograms obtained, including the chromatogram of standard JP-5, is shown in Figures 4 through 11.

The B_1 and B_2 series are duplicate studies of 16.25 µL model JP-5 added to 500 mL of basal medium; C_1 and C_2 are duplicate studies of 130 µL of model JP-5 added to 500 mL of basal medium.

TABLE 3. EXPERIMENTAL PROCEDURE FOR MODEL JP-5 BIODEGRADATION STUDY

Flask	Basal medium (500 mL)	Yeast extract (1.0 mL)	Carbon source	Inoculum
A_1	+	+	0.5 mL DBSS stock* (12.5 mg C)	1.0 mL acclimated Flask A
A_2	+	+	0.5 mL DBSS stock* (12.5 mg C)	1.0 mL acclimated Flask A
A_3	+	+	0.5 mL DBSS stock* (12.5 mg C)	None
B_1	+	+	16.25 µL JP-5 (12.5 mg C)	1.0 mL acclimated Flask B
B_2	+	+	16.25 µL JP-5 (12.5 mg C)	1.0 mL acclimated Flask B
B_3	+	+	16.25 µL JP-5 (12.5 mg C)	None
C_1	+	+	130 µL JP-5 (100 mg C)	1.0 mL acclimated Flask C
C_2	+	+	130 µL JP-5 (100 mg C)	1.0 mL acclimated Flask C
C_3	+	+	130 µL JP-5 (100 mg C)	None
D	+	+	None	0.33 mL acclimated cells from A, B, and C Flasks

*DBSS Stock - Dodecylbenzene sodium sulfonate (standard) (40.3 mg/mL) - (25 mg carbon/mL)

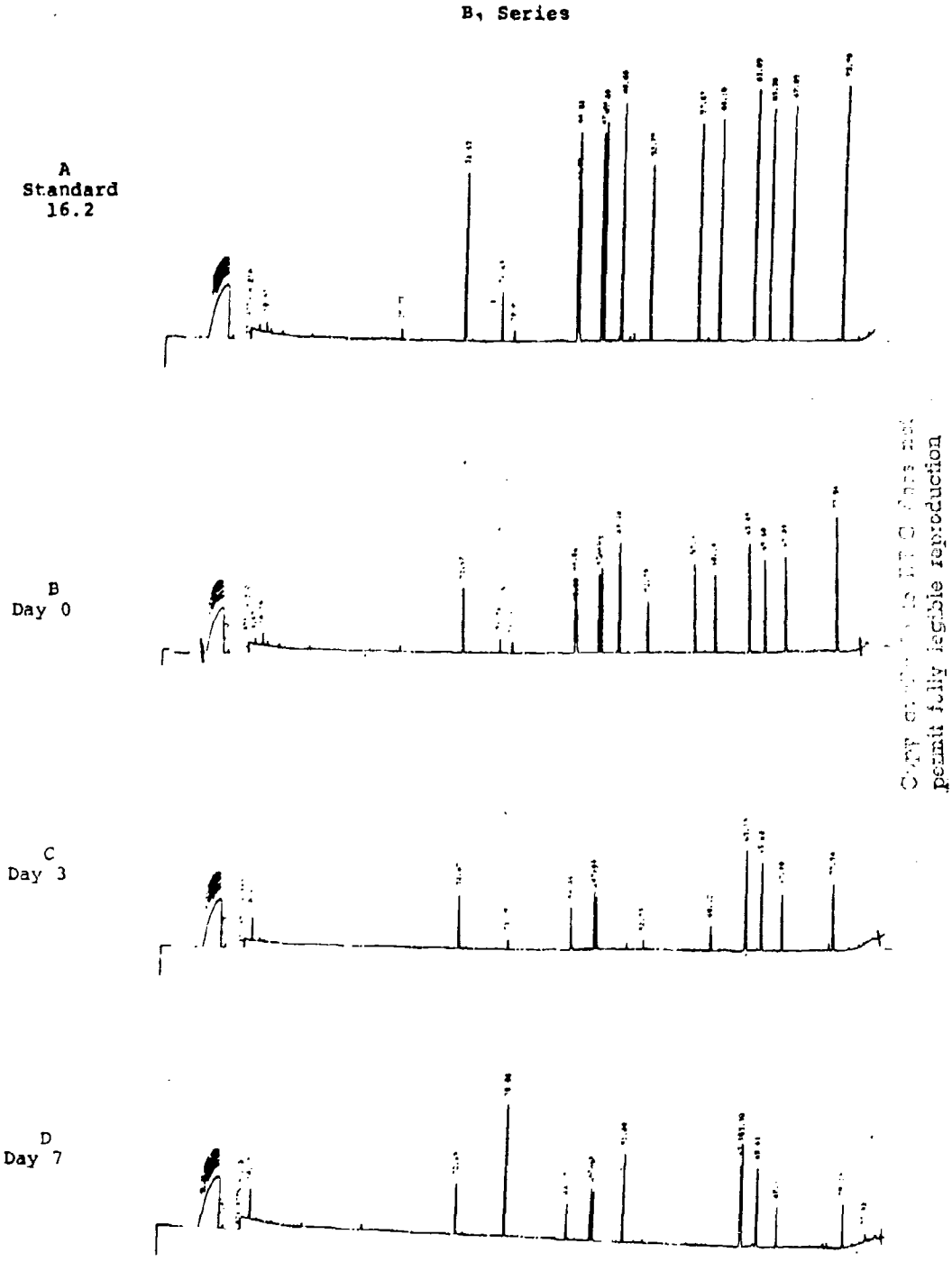

Figure 4. Gas chromatogram of pentane extracts from biodegradation of model JP-5, 16.2 µL-standard, day 0, day 3, day 7.

B_1 Series (continued)

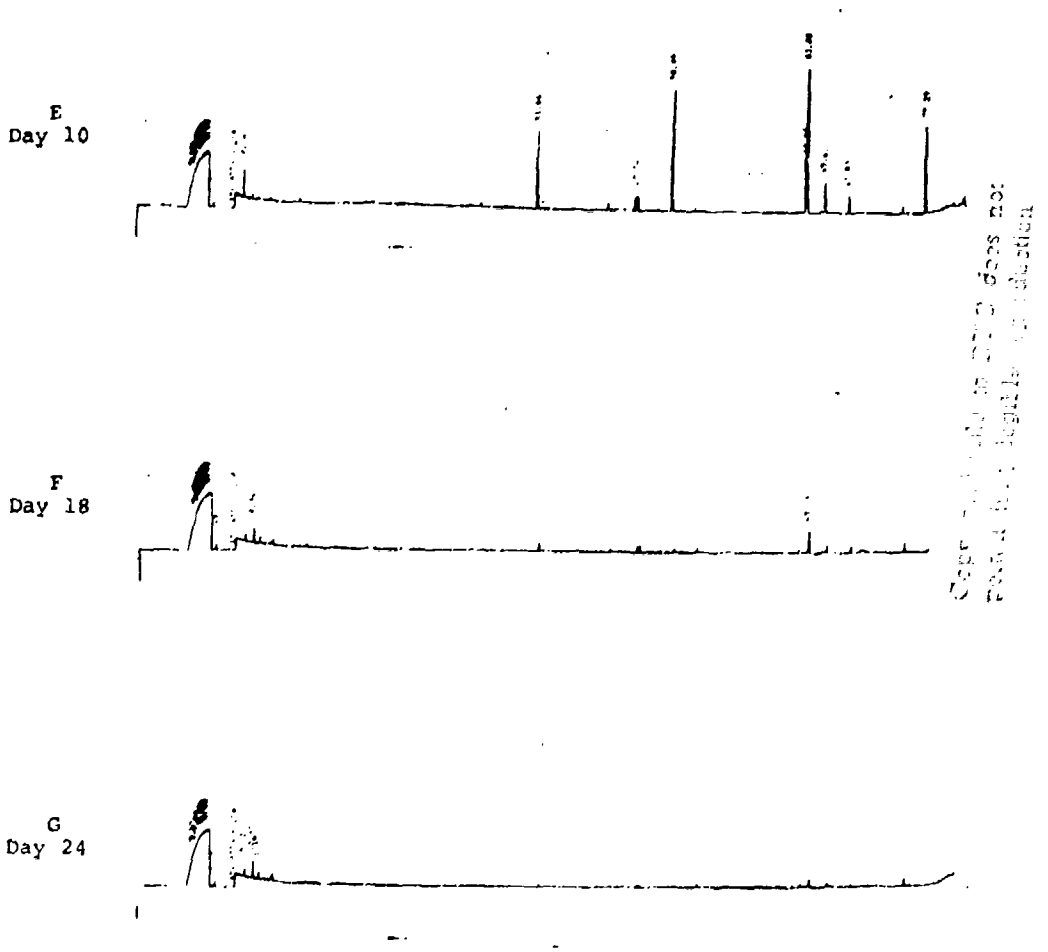

Figure 5. Gas chromatogram of pentane extracts from biodegradation of model JP-5, B_1-16.25 µL, day 10, 18, 24.

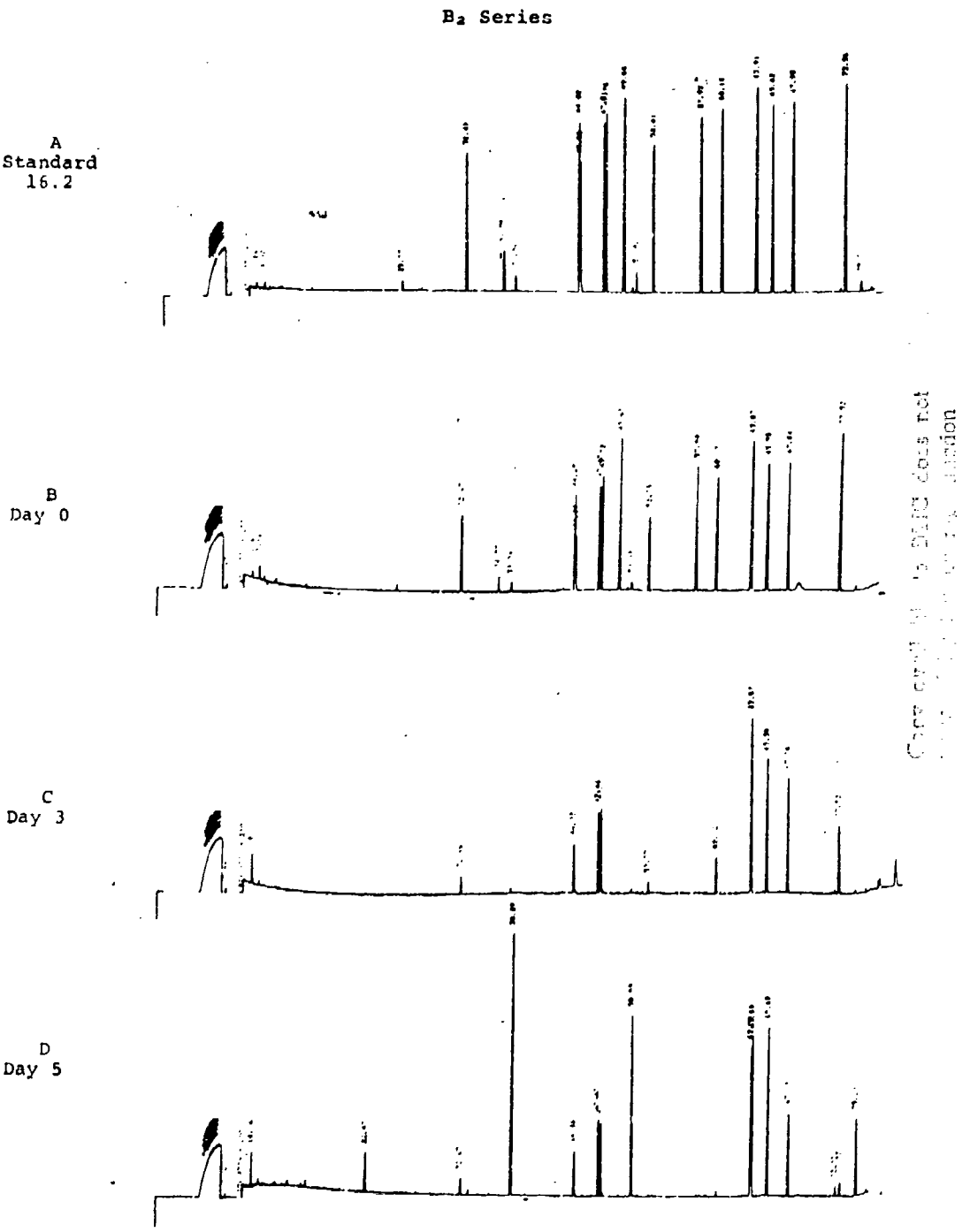

Figure 6. Gas chromatogram of pentane extracts from biodegradation of model JP-5, B_2-16.25 µL, standard, day 0, 3, 5.

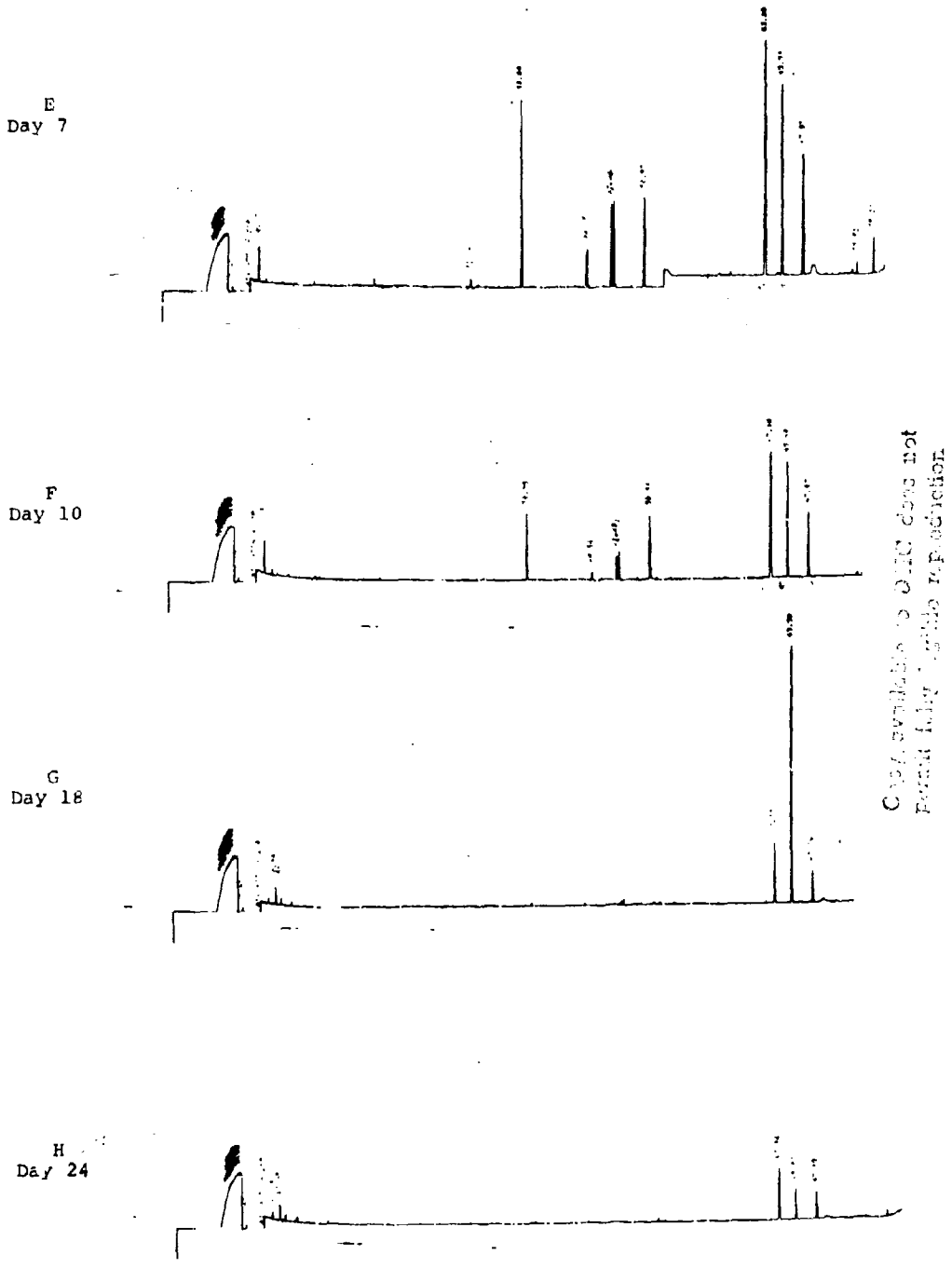

Figure 7. Gas chromatogram of pentane extracts from biodegradation of model JP-5, B_2-16.25 µL, day 7, 10, 18, 24.

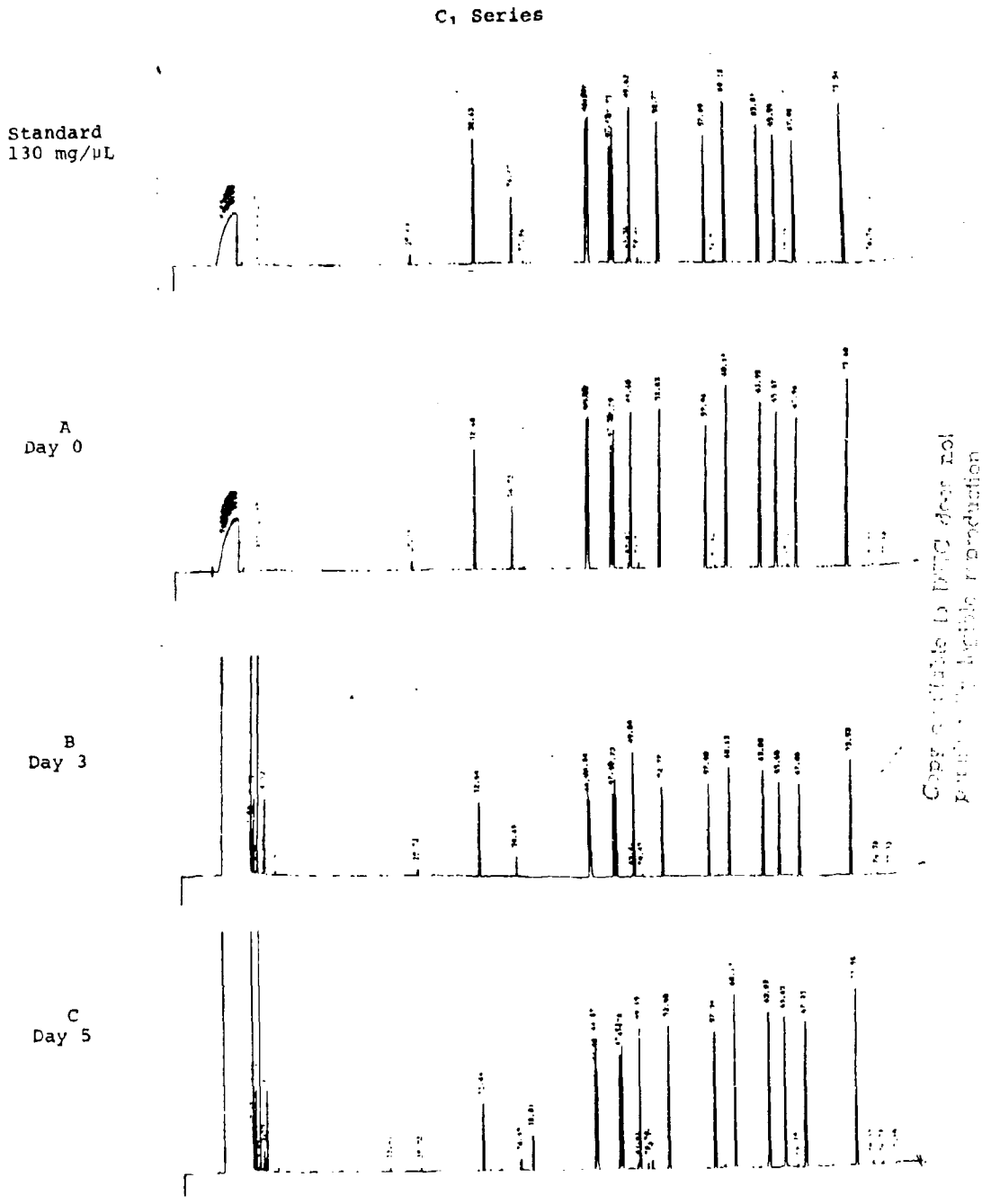

Figure 8. Gas chromatogram of pentane extracts from biodegradation of model JP-5, C_1-130 µL, standard, day 0, 3, 5.

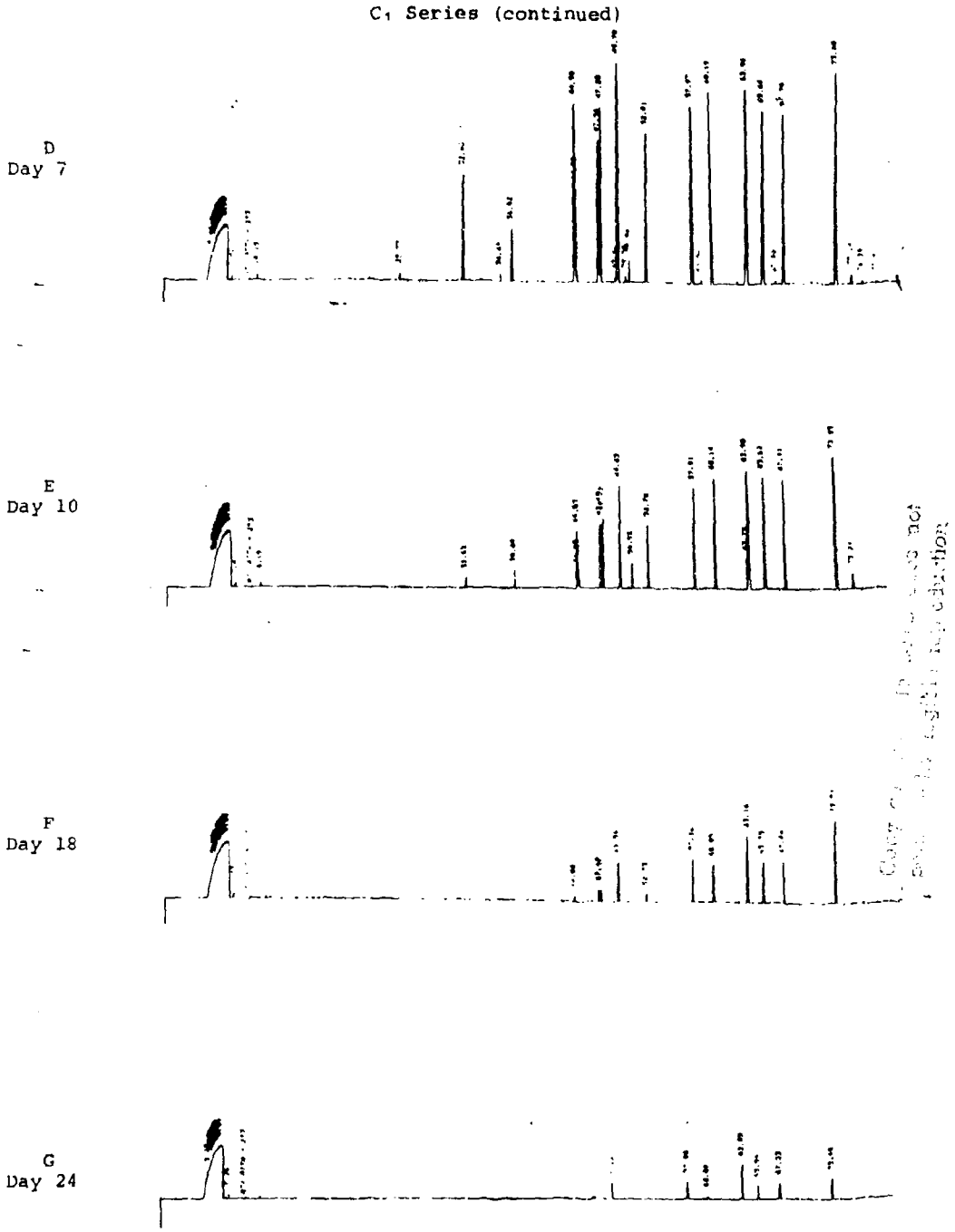

Figure 9. Gas chromatogram of pentane extracts from biodegradation of model JP-5, C_1-130 µL, day 7, 10, 18, 24.

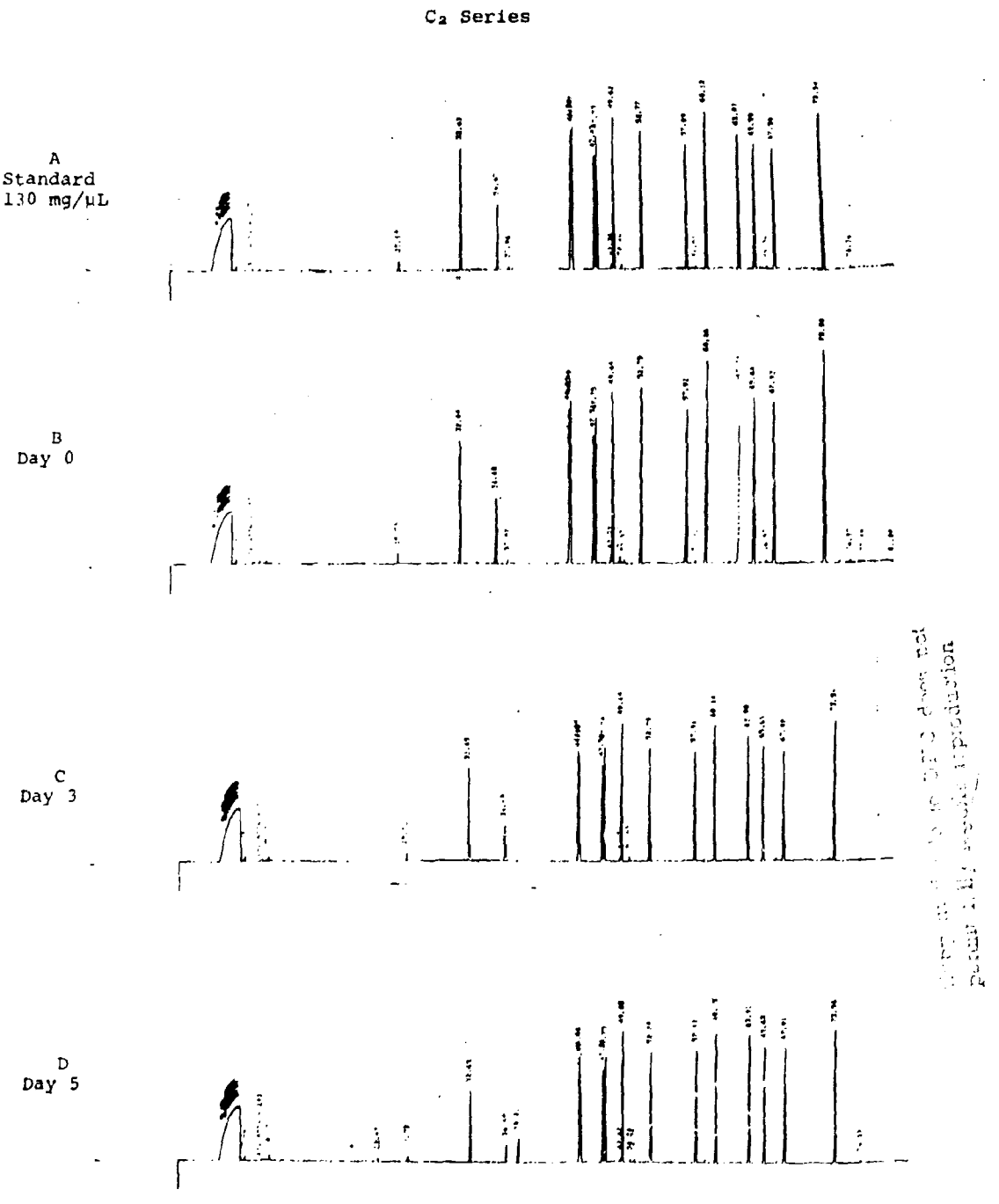

Figure 10. Gas chromatogram of pentane extracts from biodegradation of model JP-5 C_2-130 µL, standard, day 0, 3, 5.

C_2 Series (continued)

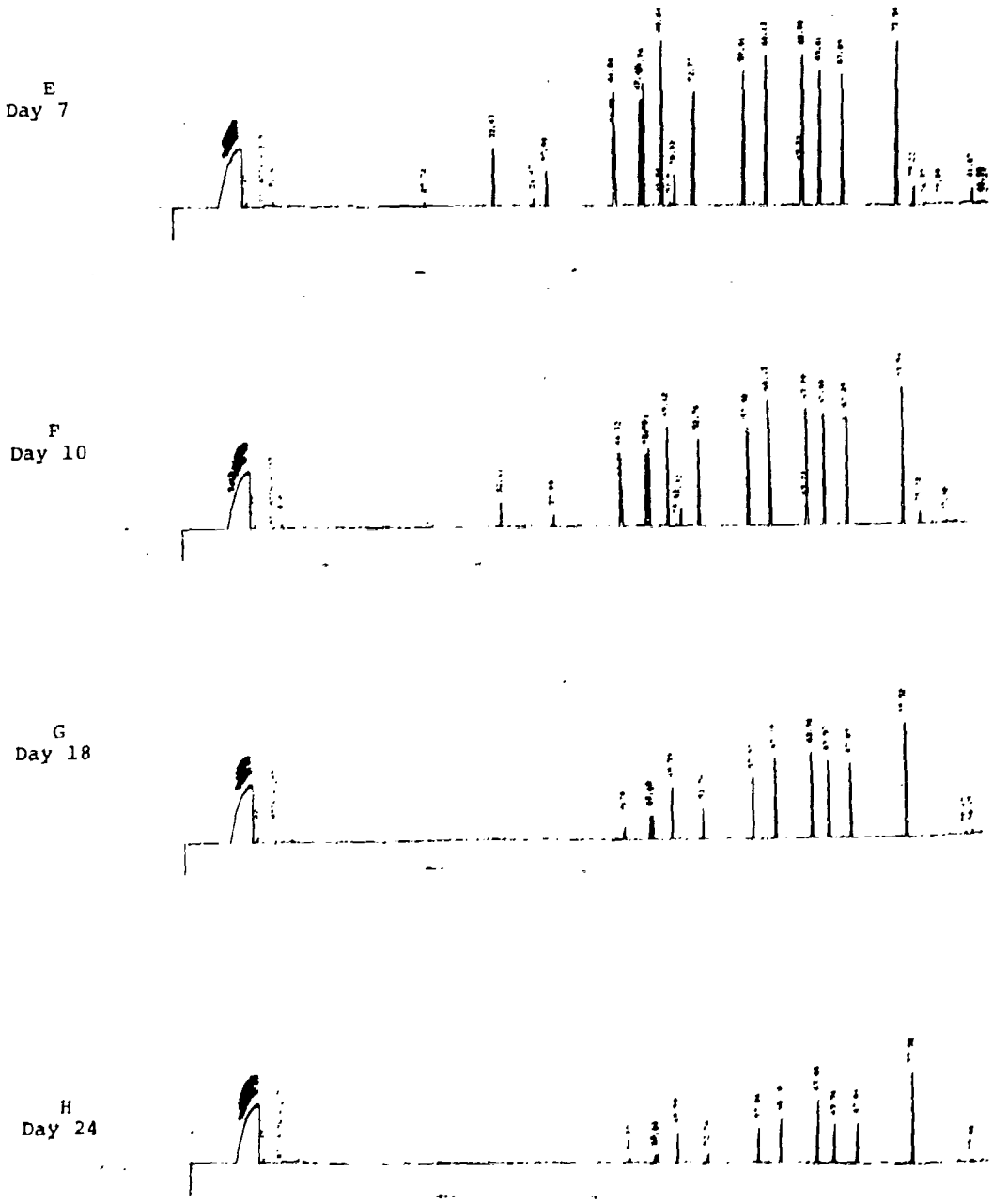

Figure 11. Gas chromatogram of pentane extracts from biodegradation of model JP-5 C_2-130 µL, day 7, 10, 18, 24.

2.2.4.3 Results

Major changes in the components of the model JP-5 took place in the twenty-five days of experimentation. Peak height ratios changed, peak heights were reduced, and extraneous peaks appeared and disappeared with time. Ultimately, total peak areas were reduced dramatically. Microbial degradation appears to play a lead role in these changes but additional experiments are being performed to determine if there is significant loss by volatility as well as by other chemical and physical factors. This study has provided hydrocarbon transformation data obtained by isolating the effects of soil microbes in reaction flasks. Similar transformations of the hydrocarbons will be assessed in the soil ecosystem as the hydrocarbon compounds progress are transported through the soil.

2.2.5 Soil Biota Population Assessment

Large numbers of small subsurface animals exist in soils, especially in the soil horizons that contain higher levels of natural organic material (i.e. the A and B soil horizons). These small animals can be used to indicate changes in the soil ecosystem brought about by foreign chemicals. For this study, two significant groups of soil animals, soil arthropods and soil nematodes, were selected for observation as indicators of the effects of chemicals on soil biota.

2.2.5.1 Arthropods

The majority of all living animals in the soil are arthropods, and a high proportion of soil biota is made up of representatives of this phylum. The most important soil arthropods are termites, beetles, ants, flies, myriapods, springtails, and mites. A relatively simple system used to isolate these small animals from soil to obtain population counts was described by Pramer and Schmidt [3]. A lightbulb is used to dry the soil sample. This causes the arthropods to migrate with the moisture until they drop through a screen supporting the soil sample and into a collecting reservoir. (See Figure 12)

Control soil samples were studied in which 160 cm^3 of soil was placed in a 500-mL, 10-cm diameter glass funnel. Cheese cloth was placed over a wire screen in the bottom of the funnel to prevent soil from falling into the collection vessel. A incandescent light bulb was placed 5 inches (12.7 cm) above the soil core sample. A collection vial containing 70% alcohol was placed under the funnel. The number of arthropods in control soil samples varied from 17-65 per soil sample.

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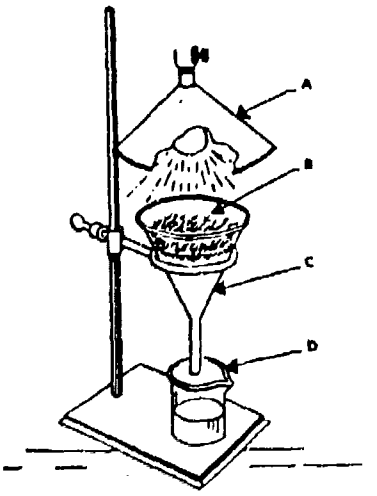
[3] Pramer, D., and Schmidt, E.L. Exercise 6 - arthropods. In: Experimental soil microbiology. Minneapolis, Burgess Publishing Company, 1964, 18-19.

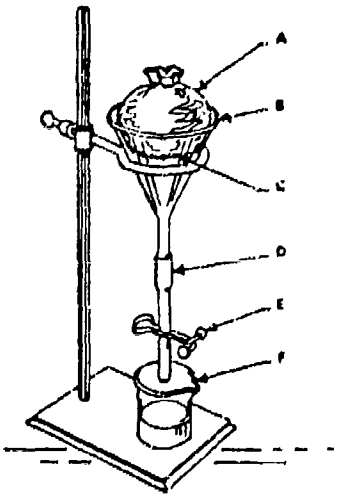


BERLESE FUNNEL FOR EXTRACTING ARTHROPODS FROM SOIL AND LITTER: A, lamp, B, screen supporting litter, C, funnel, and D, collecting vessel containing ethanol.

Figure 12. Berlese funnel for extracting arthropods from soil and litter.

2.2.5.2 Nematodes

Nematodes are round worms of the phylum Aschelminthes which inhabit soil near the ground surface and around plant roots. These animals were recovered for counting by use of the Baermann Funnel shown in Figure 13.



BAERMANN FUNNEL FOR EXTRACTING NEMATODES FROM SOIL: A, soil sample wrapped in cheesecloth secured with rubber band; B, funnel C, screen, D, rubber tubing; E, pinch clamp, and F, collecting vessel.

Figure 13. Baermann funnel for extracting nematodes from soil.

About 75 g of control soil was placed in four layers of cheese cloth that had been spread over a screen placed in the bottom of funnel. A short length of rubber tubing extending from the funnel was closed with a pinch clamp. The excess cheese cloth was placed over the top of the soil, deionized water was added to cover the soil surface. After 24 hours, 10 mL of water was collected by opening the pinch clamp. The water was placed in a Petri dish and examined for numbers of nematodes. A large variation in nematode counts per soil sample was encountered. Improved methods of identifying and counting are presently being studied.

2.2.6 Headspace Gases Analyses

Vaporization of toxic materials from soil surfaces obviously affects their fate because they escape into the air. The fate of the evaporated volatile hydrocarbon materials in jet fuels is assessed in our studies by the injection of the gases from above the soil into the gas chromatograph. Figure 14A is a chromatogram of 20 µL of headspace gas withdrawn from a core treated 7-days previously with JP-4 fuel. Figure 14B is a chromatogram of JP-4 headspace vapors taken above the liquid standard. Very small amounts of components eluted from the core head space match up closely with peaks of the standard JP-4 components. Future studies of core headspace gases will include concentration of the hydrocarbon vapors gases prior to injection into the gas chromatograph.

Headspace gases from model the JP-5 degradation studies described in Section 2.2.4 were analyzed by injecting 20-µL volumes of vapors from the control reaction flask (see Figure 15A). Figure 15B is a gas chromatogram of vapors taken from the headspace above the model JP-5 standard. The gas chromatograms shown in Figure 15 indicate that model JP-5 components evaporate readily into the headspace above the liquid. Some of the losses of components seen in the biodegradation experiments is due to volatility of these components.

2.2.7 Circular Profiling

The chromatograms reproduced in Section 2.2.4 show progressive, but many times very subtle, changes in the components of the model JP-5. Where very similar and complex sets of data are to be compared and such subtle differences noted, an optional plotting techniques, circular profiling, can aid in amplifying the differences. One data set, the C_1 series of the degradation study, was plotted using computerized circular profiling techniques [4].

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[4] Ross, W. D., Hillan, W. J., Flayler, K. A., Pustinger, J. V., Brooks, J. J., and Eisentraut, K. J. Use of circular profiling techniques in gas chromatography. Journal of Chromatographic Science. 15:461, 1977.

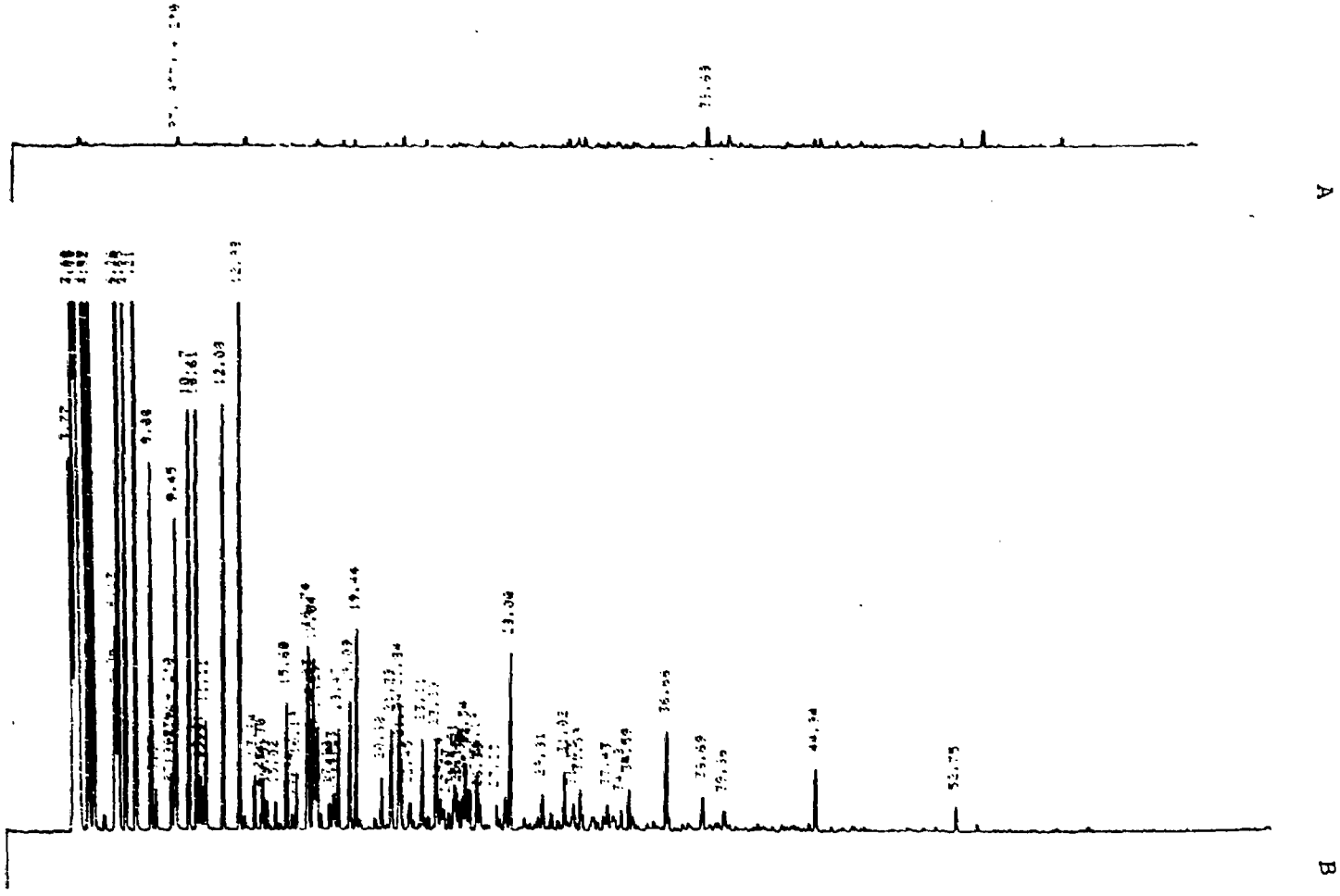

Figure 14. Gas chromatogram of headspace gases from A. JP-4 treated soil core B. chromatogram of vapors from above shale derived JP-4 standard.

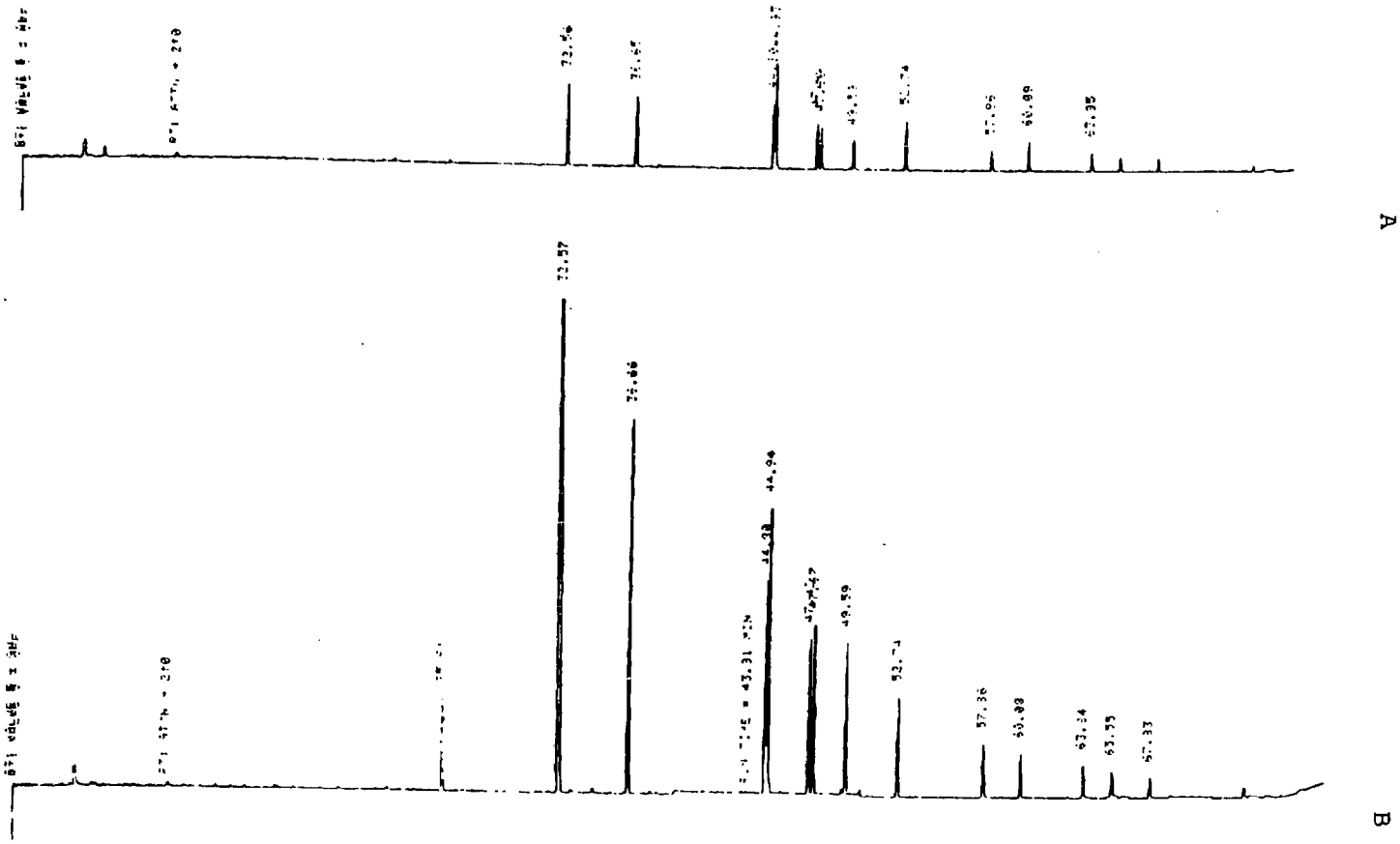

Figure 15. Gas chromatogram of headspace gases from A. Model JP-5 biodegradation reaction flask. B. chromatogram taken above model JP-5 standard.

The circular plots of the chromatograms are produced by entering retention times which relate these data to a scale factor selected to maximize the use of 360 degrees in a circular plot area. In order to maintain a consistent scale for comparison of the sequence of gas chromatograms a factor of 4 was used as a scale factor since none of the retention times exceeded 90 minutes. The chromatograms illustrate the changes in the volatile components of JP-5 caused by microbial degradation. The corresponding peak area was plotted linearly along the radii emanating from the center of the circle at the appropriate number of degrees dictated by the retention times. To maximize the use of the plot area, the area percent data were normalized to the area percent value of the largest peak in any particular data set, then multiplied by the radius of the plot area. The distinctive pattern is produced by a line originating at the center of the circle, the origin, passing through each of the data points and finally returning to the origin.

Figures 16 and 17 illustrate the circular profiles with matching gas chromatograms for model JP-5 inoculated with soil microorganisms during the biodegradation investigations. The circular profiles readily attract attention to subtle changes in the gas chromatograms as the components change with time. This is especially true of the standard profile, day zero, day three and day five biodegradation curve (See Figure 16).

2.2.8 Acute Cytotoxicity of Jet Fuels Using Mammalian Cells in Culture.

The methodology used in this study to determine the bioactivity of potentially toxic materials is the mammalian cell clonal assay technique [5]. This test is a rapid (<1 week) *in vitro* assay which used mammalian cells, both hamster (CHO-K1) and human (D98S). The CHO cell line is nearly an ideal cell line because of its high cloning efficiency (95%) and excellent colony-forming properties; i.e. it forms tightly packed cells in discrete, easily distinguished colonies that can be counted automatically. The cell line exhibits continuous cell line growth properties, is highly sensitive to toxic materials, e.g. 50% survival of formed colonies (EC_{50}) at a concentration of 0.2 µg per mL of cadmium chloride, and shows toxicity correlation with *in vivo* test systems. The CHO clonal cytotoxicity test system has been validated by the analysis of many organic compounds, environmental samples, metal salts, and known positive standards [6-10]. The CHO clonal assay method

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[5] Wininger, M. T., Kulik, F. A., and Ross, W. D. *In vitro* clonal cytotoxicity assay for chemicals using Chinese hamster ovary cells (CHO-K1), Procedure 75084. Tissue Culture Association Manual. 5(2):1091, 1979.

[6] Wininger, M. T., Kulik, F. A., and Ross, W. D. *In vitro* clonal cytotoxicity assay using Chinese hamster ovary cells (CHO-K1) for testing environmental chemicals (abstract). *In Vitro*. 14:381, 1978.

continued

described here uses a colony counting end point. Results are rapidly quantified with an automated colony counter, and data are reduced via a computer program.

Cytotoxicity assays were performed on shale-derived JP-4, petroleum-derived JP-4, and model JP-5, using both CHO cells and D98S cells. The concentrations used are shown in Tables 4 and 5. Table 4 shows processed acute cytoxicity data obtained from the computer for JP-4 (shale) using CHO-K1 cells; Table 5 is data produced by D98S cells. Figure 18 is a graph comparing cytotoxicity curves of shale- and petroleum- derived JP-4 and model JP-5 as concentration of material added to the growth medium versus the percent colony formation. Both cell types are represented, CHO-K1 and D98S. The D98S cells were most sensitive to the model JP-5 while the CHO-K1 cells were the least sensitive to this sample. There was little difference between sensitivity of the CHO (hamster) cells versus the D98S (human) to the JP-4 whether derived from petroleum or shale. In all cases, the relative toxicity (effective concentration for 50% survival, EC_{50}) was in the low range by EPA toxicity criteria [11]. Table 6 illustrates the relative cytotoxicities determined by clonal assay for several compounds. A comparison of the relative toxicities of the jet fuels tested can be readily compared to other toxic compounds.

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[7] Wininger, M. T., Hare, R. J., Brautigam, G. F., Hill, J. T., and Ross, W. D. Determination of acute cytotoxicity of elemental phosphorus (P_4) by in vitro clonal assay using Chinese hamster ovary cells (CHO-K1). Poster presentation at the 10th Annual Ohio Valley Symposium. Hueston Woods State Park, Ohio, 1978, June.

[8] Wininger, M. T., Kulik, F. A., and Ross, W. D. Short-term toxicity testing of chemicals using cultured animal cells. Ohio Journal of Science. 79:70, 1979.

[9] Campbell, J. A., Garrett, N. E., Huisingh, J. L., and Waters, M. D. Cellular toxicity of liquid effluents from textile mills. Presented at the Textile Industry Technology Symposium. Williamsburg, Virginia, 1978, December.

[10] Wininger, M. T., Hare, R. J., Brautigam, G. F., Hill, J. T., Wilson, J. D., and Ross, W. D. Determination of acute cytotoxicity of elemental phosphorus (P_4) by an in vitro clonal assay using Chinese hamster ovary cells (CHO-K1) (abstract). In Vitro. 15:199, 1979.

[11] IERL-RTP Procedures manual: level 1 environmental assessment biologial tests. Research Triangle Park, NC; U.S. Environmental Protection Agency; 1980 September. p. 62. Contract No. 68-02-2681.

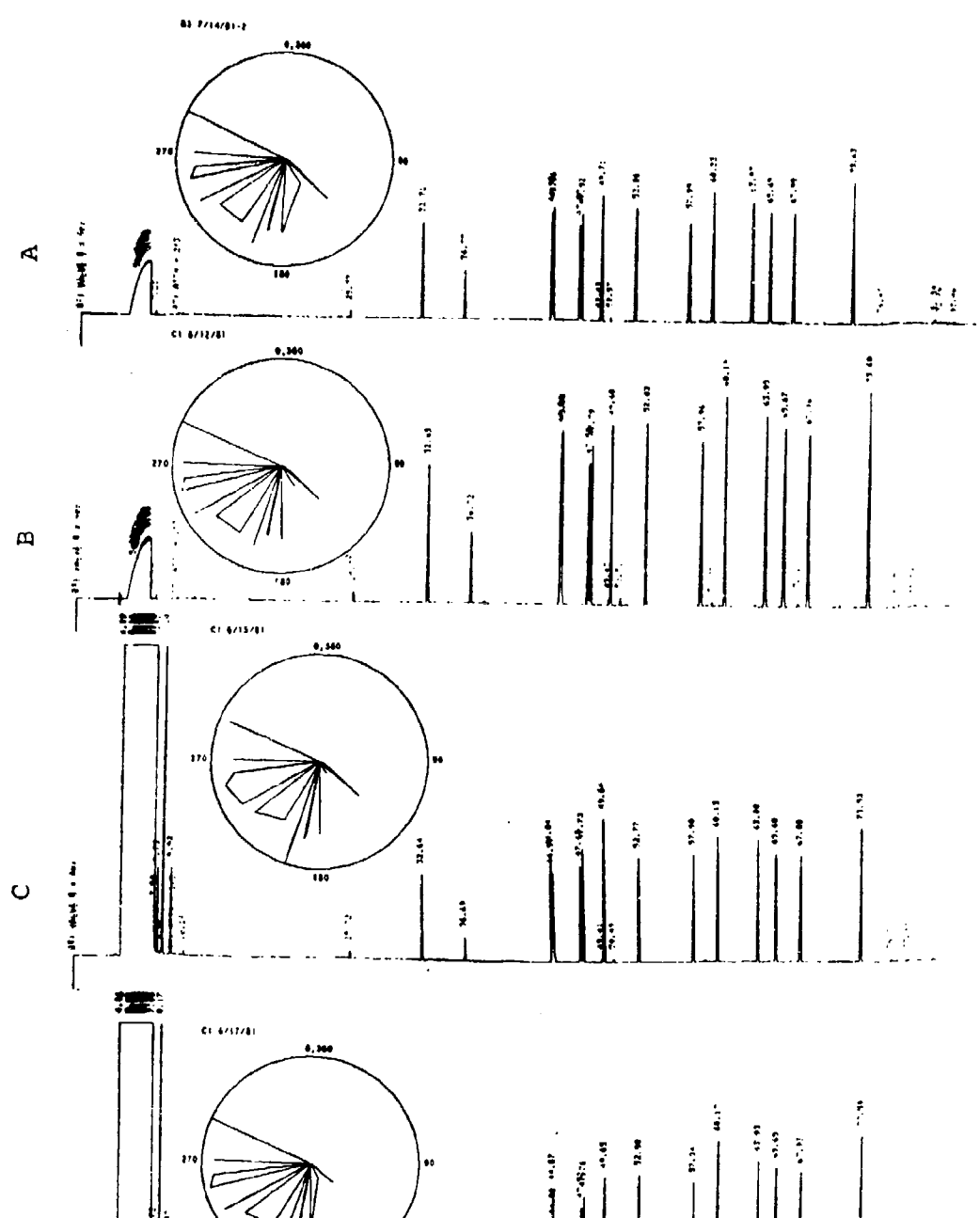

Figure 16. Gas chromatograms of model JP-5 biodegradation plotted using computerized circular profiling - C_2 standard, day 0, 3, 5.

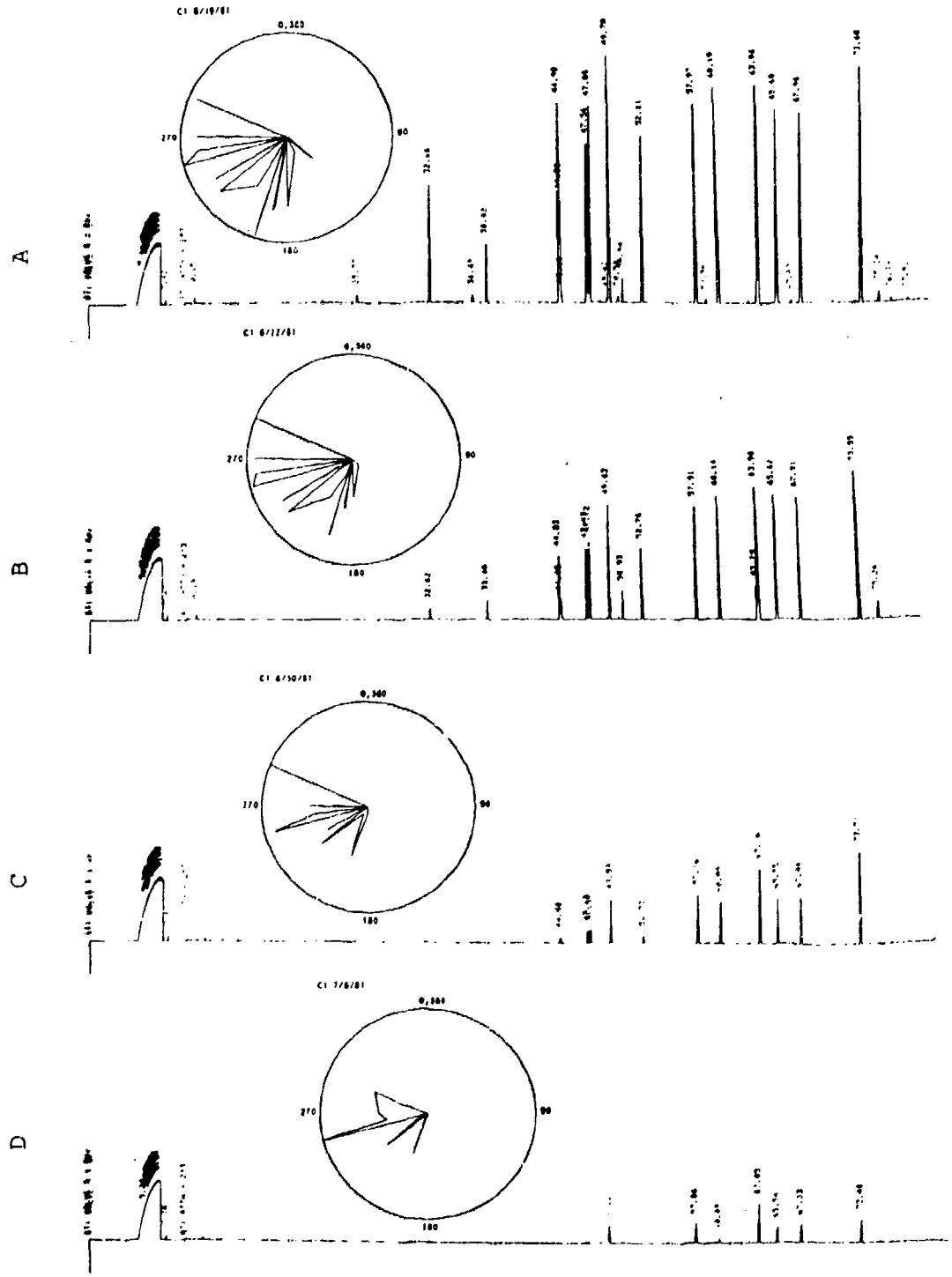

Figure 17. Gas chromatogram of model JP-5 biodegradation plotted using computerized circular profiling, day 7, 10, 18, 24.

TABLE 4. CYTOTOXICITY DATA FOR SHALE JP-4 OBTAINED WITH CHO-K1 CELLS

CYTOTOXICITY DATA FOR SHALE JP-4(F33615) 5-18-81
CELL LINE: CHO-K1 PAGE REF: -

CONTROL (BACKGROUND) VALUES	MEAN VALUE	STANDARD DEVIATION
510	498	10
485		
504		
490		
500		

CONCENTRATION (UL/ML)	REPLICATE VALUES	MEAN VALUE	STANDARD DEVIATION	PERCENT SURVIVAL
5	0 0 0	0	0	0
1	0 0 0	0	0	0
.8	0 0 0	0	0	0
.6	14 11 0	8	7	2
.2	428 479 460	456	26	92
.1	498 471 495	488	15	100

BASIC
>

TABLE 5. CYTOTOXICITY DATA FOR SHALE JP-4 OBTAINED WITH D98S CELLS

CYTOTOXICITY DATA FOR SHALE JP-4 (F33615) 5-18-81
CELL LINE: D98S PAGE REF: -

CONTROL (BACKGROUND) VALUES	MEAN VALUE	STANDARD DEVIATION
280 295 294 296 288	291	7

CONCENTRATION (UL/ML)	REPLICATE VALUES	MEAN VALUE	STANDARD DEVIATION	PERCENT SURVIVAL
5	0 0 0	0	0	0
1	0 0 0	0	0	0
.8	0 0 0	0	0	0
.6	0 0 50	17	29	6
.2	310 312 268	297	25	100
.1	287 297 315	300	14	100

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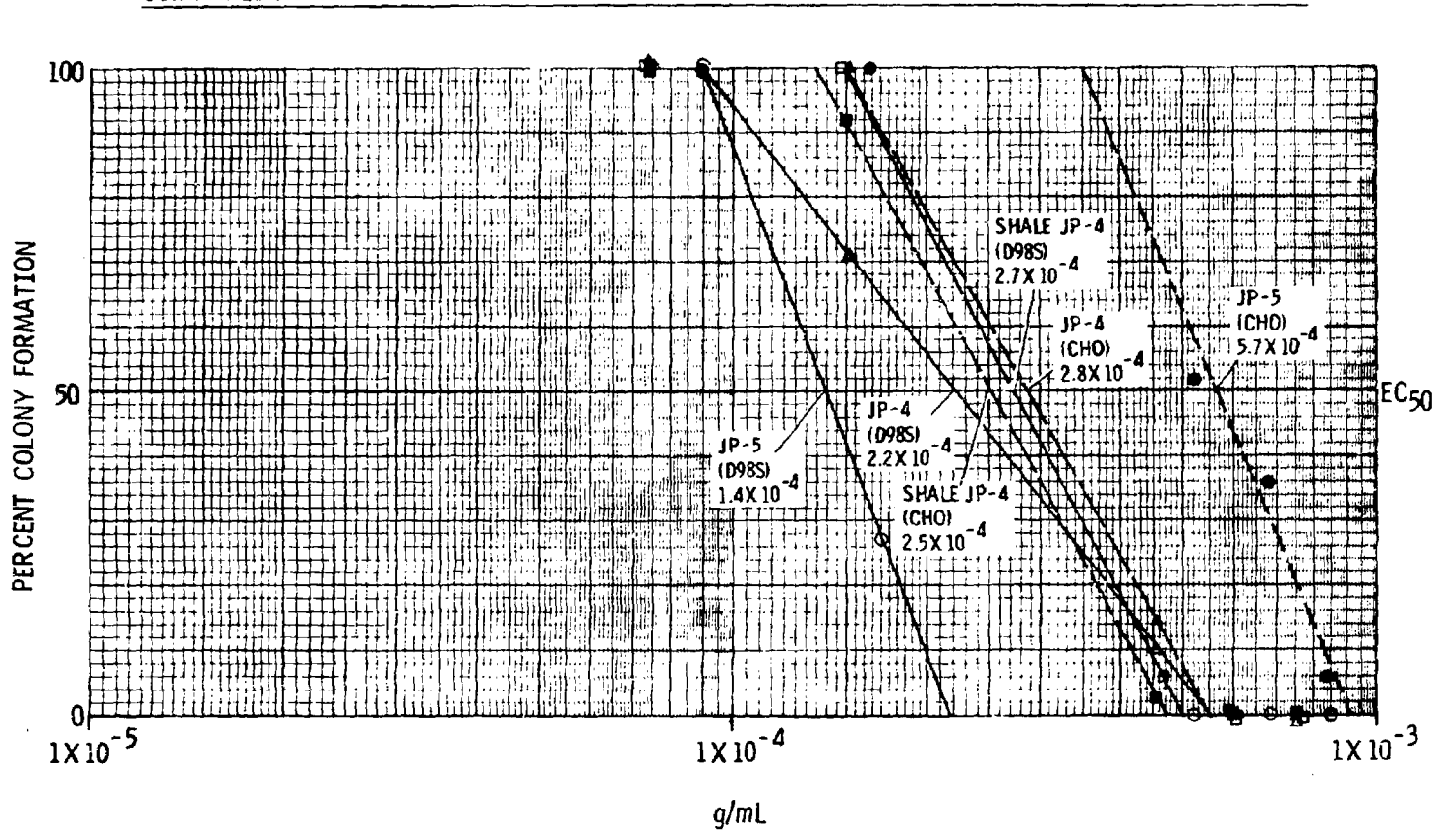

Figure 18. Comparison of cytotoxicities of jet fuels to CHO (Chinese Hamster)

TABLE 6. RELATIVE CYTOTOXICITIES DETERMINED BY MAMMALIAN CELL CLONAL ASSAY

	CHO EC_{50} (g/mL)	CHO EPA toxicity rating	D98S EC_{50} (g/mL)	Whole animal LD_{50} (mg/kg)
Cadmium chloride	1.6×10^{-7}	high	2×10^{-7}	3 - 24
Potassium cyanide	4.8×10^{-7}	high	9×10^{-8}	4 - 16
Mercuric chloride	2.9×10^{-6}	high	5×10^{-7}	5 - 120
Hydroxylamine hydrochloride	3.2×10^{-5}	moderate	7×10^{-5}	100 - 400
Tetralin	5×10^{-5}	moderate	$6 \times 10^{-}$	-
Biphenyl	$>1 \times 10^{-4}$	low - very low	4×1	56
Phenol	1.4×10^{-4}	low	$1 \times$	250 - 415
Ethyl benzene	1.7×10^{-4}	low	-	-
Shale oil-derived-JP-4	2.5×10^{-4}	low	2.7×10^{-4}	-
Petroleum-derived-JP-4	2.8×20^{-4}	low	2.2×10^{-4}	500
Chlorobenzene	5.5×10^{-4}	low	-	-
JP-5 (model)	5.7×10^{-4}	low	1.4×10^{-4}	-
Phenyl ether	6.5×10^{-4}	low	-	-
Aniline	7.1×10^{-4}	low	-	200 - 1250
Chloroform	1.4×10^{-2}	very low	-	700 - 1650

2.3 EVALUATION OF JP-4 JET FUEL USING THE TERRESTRIAL ECOSYSTEM

A study was performed to evaluate the fate of shale-derived JP-4 in the terrestrial ecosystem and other associated evaluation systems.

2.3.1 Preparation of Soil Cores

Two soil cores were prepared by techniques described earlier [1]. Cores were taken from soil on 18 May 1981 in Preble County, Ohio, Township 7, North Range 1, East, Section 17, N.W. corner, grassland, untilled, Reesville series soil type. The core dimensions are: ecosystem 9 (control), 36.75 cm x 10 cm; ecosystem 10 (test), 48.26 cm x 10 cm. Ecosystem 9 had three sampling probes placed 10 cm apart vertically. Ecosystem 10 had four probes placed 10 cm apart vertically. Each probe was placed in the core at varying distances from the outside wall of the ecosystems. Sample boats used in core 10 were filled with XAD-7, previously shown to recover components of JP-4 [1].

The cores were placed vertically in a windowless laboratory subject to only artificial fluorescent lighting for 8.5 hours per day. The laboratory temperature was ~21°C to 25°C. The walls of the ecosytem were covered on the outside with black polyethylene film up to the soil surface. The caps were in place to confine the headspace for carbon dioxide monitoring and hydrocarbon vapor analyses. Potassium hydroxide traps were placed in the headspace above the soil to trap CO_2. The cores were watered with deionized water at a rate of 100 mL biweekly by the water distribution system shown in Figure 1.

2.3.2 Carbon Dioxide Measurements

The carbon dioxide traps were removed and titrated every 2-4 days. The assessment began as soon as the cores were brought into the laboratory in order to determine a pre-experimental equilibration time. This equilibration time provided a pre-test period for the soil cores and the associated biota to adapt to the new conditions of the laboratory. Figure 19 is a graph of carbon dioxide in the headspace for two ecosystems, filled with soil cores, namely, ecosystem 9 control core and ecosystem 10 test core. The curves indicate accumulated or total amounts of CO_2 evolution.

A very steady efflux of CO_2 in both cores is indicated by the curves. Slightly larger amounts of CO_2 evolved from control core 9. The curves show that an adjustment period was not required by these two cores. The test core 10 was treated with 10 mL of JP-4 jet fuel on day 36 after the cores were brought to the laboratory. Within five days, the CO_2 evolution increased from this core, crossed the control evolution curve within 2 weeks, and continued to increase. This distinctive and steady increase in CO_2 evolution could be interpreted as increased biota respiration activity,

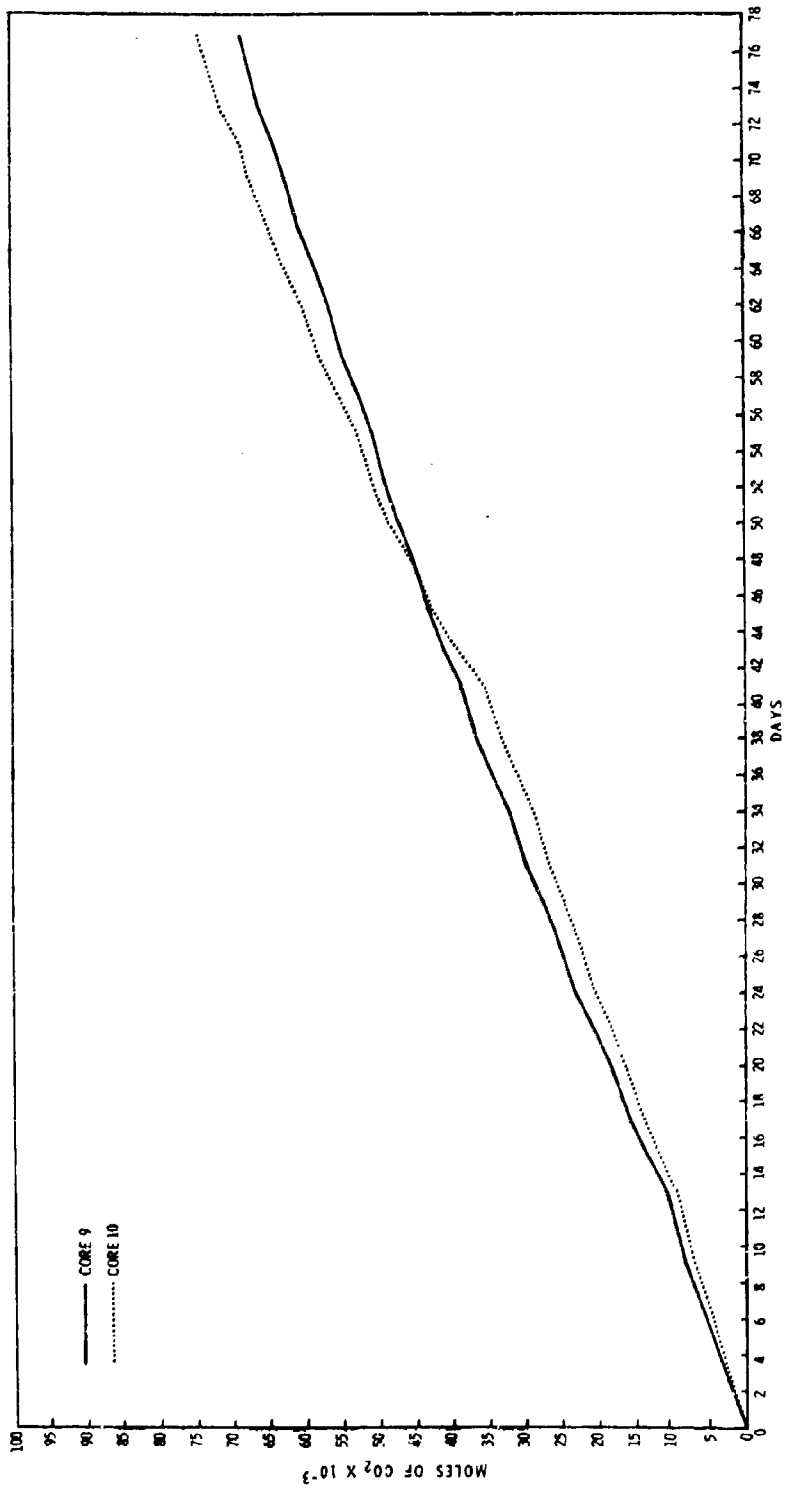

Figure 19. Comparison of carbon dioxide evolution in soil core 9 and 10 headspace.

suggesting that the shale-derived JP-4 fuel is supplying a carbon source for the soil biota. This data could also be interpreted as a biota stress symptom or a change in species populations resulting from destruction of more vulnerable species. This theory will be substantiated if the CO_2 decreases rapidly in subsequent test days.

2.3.3 Treatment of Test Core with Shale-Derived JP-4 Jet Fuel

The test core was treated with 10 mL of shale-derived JP-4 by applying the fuel directly to the soil surface to mimic a spill of the material on land surface. The 10 mL application is equivalent to a spill of 1.27 liters per square meter. A biweekly application of 100 mL water was used to simulate an equivalent of ~13 inches of rain per year. This volume of rainfall was selected by assuming about one third of a 40-inch rainfall per year (western Ohio average rainfall) percolates into the soil while two thirds evaporates or runs off.

2.3.4 Leachate Recovery

Leachates were recovered in French square bottles attached to the sampling probes by Teflon tubing and volumes measured. The leachate eluting from the bottom was also collected and measured. Table 7 shows the amounts of leachate recovered from probes and the bottom of the ecosystem.

2.3.5 Recovery of Hydrocarbon Compounds by XAD-7 in Core Sampling Probes

The sampling probes were filled with 1.25 cm^3 of XAD-7 resin per probe. The adsorbent was removed from each probe every two weeks and extracted with 5 mL of pentane. Two microliters of the pentane solvent was analyzed by gas chromatography using the instrument conditions described in Table 1.

This experiment is incomplete and it will continue into the next contract year. At the cut-off time for this Annual report, three extracts from each probe had been made: days 14, 28, 42. Only the extracts from XAD from collection position one located ten centimeters below the soil surface, showed hydrocarbons in the gas chromatograms (see Figure 20). These three extracts varied considerably from each other and from the chromatogram of the starting material. The following general observations were made:

- Numbers of components are reduced in the XAD recovered hydrocarbons.
- Certain peaks are noticeably increased in the extract of XAD recovered on day 10. (Figure 20A).

TABLE 7. LEACHATE RECOVERED FROM ECOSYSTEMS IN MILLILITERS

Week	Date	Core 9 (control) Probes				Core 10 (Test) Probes				
		1	2	3	B	1	2	3	4	B
1[a,b]	6/1/81	0	1	0	13	0	0	0	0	0
2	6/8/81	0	0	0	1	0	0	0	0	0
3[a,b]	6/22/81	25	0	0	19	0	0	0	0	10
4	6/29/81	1	2	0	0	0	0	0	0	0
5	7/6/81	0	0	0	0	0	0	0	0	0
6	7/13/81	0	0	0	0	0	0	0	0	0
7	7/20/81	30	22	0	0	8	0	0	0	0
8[a,b,c]	7/29/81	50	11	0	0	20	0	0	0	0
9	8/3/81	0	9	0	8	14	0	0	0	18
10[a,b]	8/10/81	59	10	-	8	51	0	0	0	25
11	8/17/81	-	-	-	-	-	-	-	-	-
12	8/24/81	50	8	0	3	66	0	0	0	2
13	8/31/81	16	9	0	6	7	0	0	0	0
14	9/7/81	67	64	0	1	33	0	0	0	7

[a]Cores were watered in the middle of the week with milli RQ water.

[b]XAD resin was removed from all positions on the same day the cores were watered.

[c]Dosed core 10 only with 10 milliliters of JP-4.

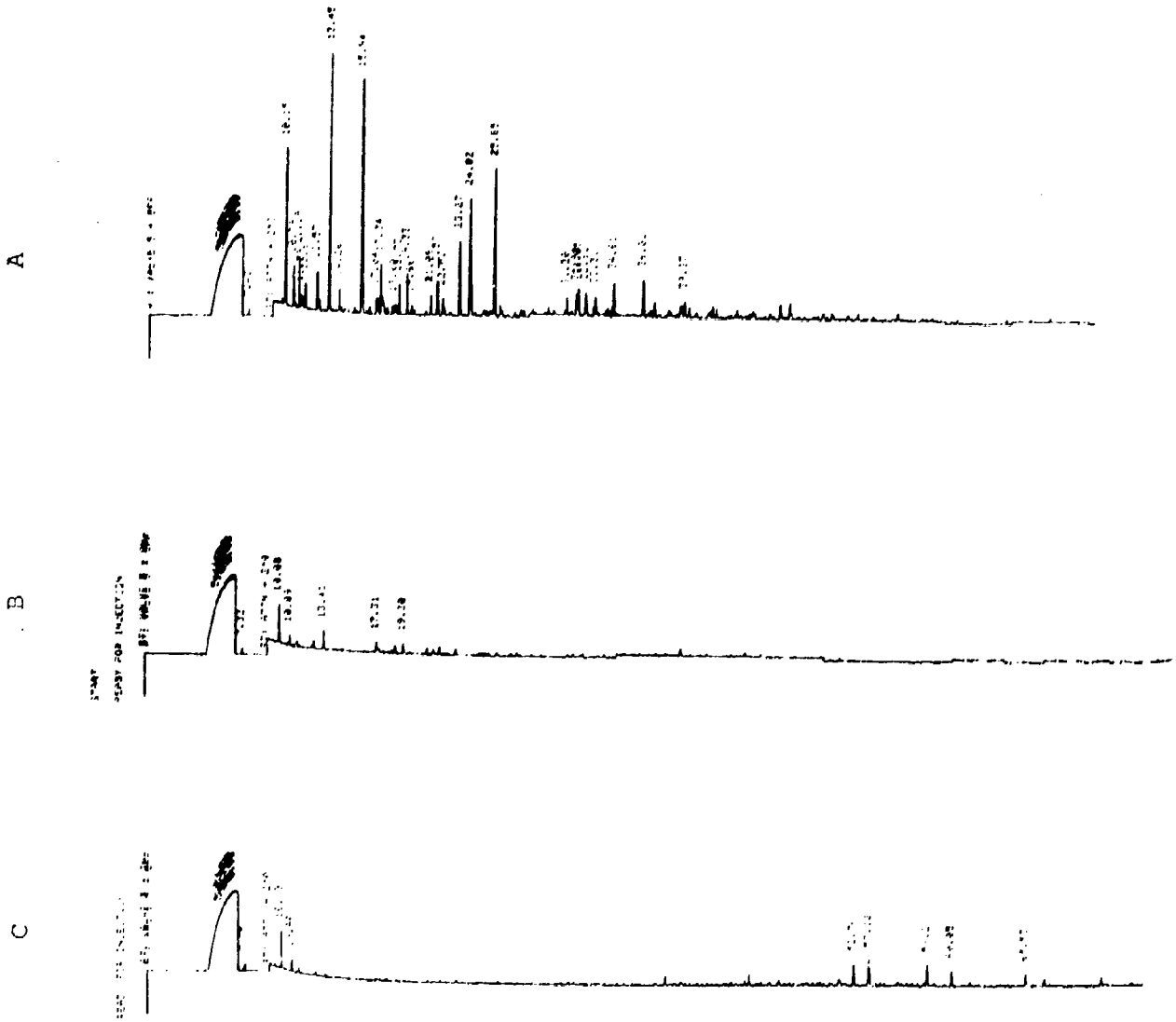

Figure 20. Gas chromatograms of XAD pentane extract from position 1,

A-Day 14
B-Day 28
C-Day 42

- Components with long retention times begin to elute on day 40 which were not present on day 16, (Figure 20 C).

2.3.6 Summary and Conclusions From Shale JP-4 Study

The data acquired on the fate of shale-derived JP-4 in the soil ecosystem includes headspace analysis, leachate transport, CO_2 evolution, transport of JP-4 components via leachate, changes in hydrocarbon content as the fuel is transported, and acute toxicity of the starting sample. The following summarizes the results obtained:

- Leachate transport through the cores is not consistent; note the variations in data presented in Table 7.

- Carbon dioxide evolution is affected by treatment of test core 10 with shale JP-4; i.e., an increase in evolution of CO_2 was noted. (See Figure 19).

- Carbon dioxide evolution from both cores was steady as soon as the cores were brought into the laboratory i.e. no equilibration time was required for adaption of the cores to laboratory conditions.

- Some shale-derived JP-4 was transported into the soil core, but only to a depth of 10 cm as is indicated by the gas chromatograms shown in Figures 20. A considerable number of changes in components occurred during the 10 cm transport through the soil cores.

- No hydrocarbons were eluted from probe positions 2, 3, 4.

- No shale-derived JP-4 was found in the bottom leachate indicating no transport through 48.26 cm. of soil core after 40 days of experimentation.

- Headspace gas analysis indicated some volatility of shale JP-4 above the treated cores (see Figure 14A)

- The acute toxicity of shale- and petroleum-derived JP-4 was found to be low by EPA criteria using the mammalian cell clonal assay.

- Biodegradation experiments with standard components (i.e. model JP-5) indicate microbial growth and utilization of the hydrocarbon components as a carbon source.

All of the above end points of the shale JP-4 fate and biological consequence investigation imply the following conclusions:

- Shale JP-4 is a material of low toxicity to mammalian cells in culture and is non mutagenic [1] at the concentrations and under the conditions tested. Because of the small amounts of organic material recovered on XAD resin in sampling probes at the various soil depths, toxicity testing was not feasible.

- Some changes in components at the position one collection point were indicated by the gas chromatographic analyses of XAD extracts. These changes may possibly be from microbial metabolism, volatilization selective adsorption, and chemical degradation.

- The increase in carbon dioxide evolution into core headspace following treatment of the test core indicates an alteration in the ecology of the ecosytem by the shale-derived JP-4.

- The variations in leachate output from the same probes and output from different probes at different times indicate a highly changing pattern of biota burrows in the soil. This becomes quite apparent when the soil is removed from the ecosystem (see Figure 21). The significance of this data is that even if contact with soil alters or destroys a material during percolation by adsorption or degradation, one open burrow from the soil surface to groundwater depths could shunt intact quantities of the spilled material to a groundwater supply. Deep soil cracks due to very dry soils could also cause channeling. Even water-insoluble materials could be forced through these channels. However, channeling through burrows to the bottom of the core did not occur in this JP-4/core 10 study.

- The headspace analysis for volatile compounds indicates a potential problem with air contamination in the immediate area of a spill of JP-4, especially with the lower boiling and more volatile components.

Figure 21. Soil removed from ecosystem after several months in the laboratory.

3. SUMMARY AND FUTURE RESEARCH PLANS

In the soil core ecosystem developed to investigate the fate of chemicals in soils, the objectives were to mimic a real world condition if materials foreign to soils and groundwater were spilled or applied to the soil surface. Whenever possible conditions are being used to provide the same variables that would be found at a particular site of a spill so that predictions can be made before an accident occurs as to the potential effects of foreign materials on various ecosystems and ultimately on health effects to humans.

With data accumulated from the first two years of research, we can now recognize some of the artifacts created by inside laboratory experiments with the ecosystems which may limit the validity of experimentation. The variables that may have significant effects but which are difficult to mimic in the labortory are:

- Outside ambient temperatures, which vary continually throughout the day and seasons.
- The system as designed to date primarily studies vertical transport of materials and is not an adequate design to study lateral diffusion effects.
- Artificial light has been used; it does not properly mimic the true effects of sunlight.
- A closed system above the soil surface is used to recover hydrocarbon volatiles and carbon dioxide. This creates artifacts limiting the true effects of ambient oxygen and carbon dioxide found under natural conditions.
- Rainfall is simulated by addition of water at a regular time sequence and in measured equal aliquots. This does not mimic the random time and amounts of rainfall encountered in nature.

The next year of research will emphasize the following studies:

- Ecosystems will be placed outside and in the ground to better simulate outdoor conditions. Recovered data from these test systems will be compared to the data from in-laboratory systems.
- Larger diameter ecosystems will be evaluated to better study lateral diffusion of materials.
- Data now being recovered will be processed by a computer program to determine correlation factors.
- Additional materials will be evaluated, e.g. model JP-5, hydrazines, and various hazardous waste materials.

4. COMMUNICATIONS RELATED TO CONTRACT

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Ross, W. D., Wininger, M.T., and Hillan, W. J. Use of cultured mammalian cell techniques to evaluate the toxicity of chemicals transformed in laboratory terrestrial ecosystems. Presented at the 32nd Annual Meeting of the Tissue Culture Association, Washington, DC 1981 June 9. Abstract 98, In Vitro, 17(3): 224, 1981 March.

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